Institut für Veterinärbiocheme und Molekularbiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Ulrich Hübscher

Arbeit unter wissenschaftlicher Betreuung von Dr. sc. nat. Julia Dorn

Regulation of Human DNA Polymerase β by Protein Kinase C Mediated Phosphorylation

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Sarah Luisa Wyck

Tierärztin

von Lörrach, Deutschland

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Prof. Dr. med. vet. Ulrich Hübscher, Referent

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1 Zusammenfassung

DNA Polymerase (Pol) β ist das wichtigste Enzym in der Basen Exzisions Reparatur (BER). Es wurde gezeigt, dass posttranslationale Modifikationen wie Phosphorylierungen einen Einfluss auf die Stabilität, Aktivität, Bindung zu Interaktionspartnern sowie die zelluläre Lokalisation von Proteinen haben. Es ist zudem bekannt, dass die Protein Kinase C (PKC) Pol β phosphoryliert, durch Phorbol-12-myristat-13acetat (PMA) stimuliert und durch ro 32-0432 inhibiert werden kann. Im Rahmen dieser Dissertationsarbeit wurde die Bindung von Pol β nach Phosphorylierung durch PKC zu zwei wichtigen BER Interaktionspartnern untersucht. X-ray repair cross complementing protein 1 (XRCC1) ist ein Gerüstprotein in der "short-patch" BER. Es ist bekannt, dass die Interaktion zwischen Pol β und XRCC1 den Komplex stabilisiert und die Aktivität von Pol β in der BER stimuliert. Um den Effekt der Phosphorylierung zu untersuchen wurde die Bindung, von Pol β WT und verschiedenen nicht phosphorylierbaren Pol β Mutanten (T10A, S30A, S44A, S55A, T67A, S180A) an XRCC1, untersucht. Es konnten Unterschiede in den Bindungskapazitäten von Pol β WT zu XRCC1 verglichen mit den Pol β Mutanten zu XRCC1 gefunden werden. Die Daten lassen vermuten, dass Pol β S55A effektiver an XRCC1 binden kann als Pol β WT. Die fünf restlichen Pol β Mutanten interagierten jedoch weniger gut mit XRCC1. Es scheint, dass die Phosphorylierung von Pol β an S55 zu einer verstärkten Bindung zu XRCC1 führt. Schliesslich ist die Interaktion von Pol β WT zu Proliferating Cell Nuclear Antigen (PCNA) wichtig für den "long-patch" BER. Die Phosphorylierung von Pol β führte zu einer verminderten Bindung von Pol β zu PCNA.

Keywords: human DNA polymerase β, Protein Kinase C, Basen Exzisions Reperatur, Posttranslationale Modifikationen

2 Summary

DNA polymerase (Pol) β is an important repair enzyme in base excision repair (BER). Posttranslational modifications like phosphorylation have been shown to have influence on the activity, stability, binding to interaction partners or subcellular localizations of proteins. It is known, that Pol β is phosphorylated by protein kinase C (PKC), which itself is stimulated by phorbol-12-myristat-13acetat (PMA) or inhibited by ro 32-0432. In this thesis work, the influence of Pol β phosphorylation on the binding to important BER proteins was tested. X-ray repair cross complementing protein 1 (XRCC1) is a scaffold protein in short-patch BER. It is known, that the interaction of Pol β to XRCC1 stabilizes the complex and enhances the activity of Pol β during BER. To investigate which effect phosphorylation of Pol β has, the binding of Pol β WT and six phosphoablated Pol β mutants (T10A, S30A, S44A, S55A, T67A, S180A) to XRCC1 was investigated. The mutant S55A appears to bind more efficiently to XRCC1 than Pol β WT, whereas the other five mutants bind less to XRCC1. In summary it seems, that phosphorylation of Pol β WT leads to an increased binding of Pol β WT to XRCC1. Finally, the interaction of Pol β with proliferating cell nuclear antigen (PCNA) is important for long-patch BER. In this case, phosphorylation and thus activation of Pol β by PKC leads to decreased binding of Pol β to PCNA.

Keywords: human DNA polymerase β, protein kinase C, base excision repair, posttranslational modification

3 Introduction

Adapted and modified from reference (1).

Deoxyribonucleic acid (DNA), a highly complex molecule, is the basis for existence of almost all forms of life. DNA contains the information relevant for life and destiny of every single cell, regardless which tissue or which function will result out of that one cell. Every organism starts from a single cell stadium out of which a whole multicellular organism can arise by cell duplication. Thus, duplication of cells is an absolute requirement for the persistence and continuation of life. The duplication of cells occurs in a cyclic fashion, and is termed the cell cycle. The human and animal bodies consists of around 10^{13} cells, which means that many cell divisions are required to generate an entire organism (2). To achieve a fully functional body, all cells have to duplicate and separate their information correctly. Such a huge number of cell divisions bear many possibilities for mistakes. The aim of cell division is, that after one round of cell cycle two cells are generated, which contain exactly the same genetic information. If mistakes happen during cell division, the integrity of the genetic information is compromised. Mutations may cause severe consequences for the single cells or even for the entire organism. Therefore the cells attempt to pass on their genetic information with as few mistakes as possible.

3.1 DNA damage and cell cycle

The cell cycle is divided in four phases. The first is G1 (gap 1), where the cell is active, followed by the S (synthesis) phase, where the entire DNA is duplicated. In the G2 (gap 2) phase the cell prepares for the subsequent separation, which takes place in the M (mitosis) phase. Mitosis is the phase in which the cell separates and, as a result two identical daughter cells arise (3). Apart from these four phases a fifth phase exists called the G0 (gap 0), in which the fully differentiated cells remain.

Several checkpoints have been integrated into the cell cycle to guarantee the fidelity of every cell division. The checkpoints monitor the integrity of the DNA, because it is very important that only correctly duplicated DNA is transmitted to the daughter cells. DNA damage sensor proteins recognize DNA strand breaks, replication errors, or other mistakes in the chromatin structure (4) and stimulate further signal transducer proteins, finally leading to activation of different effector pathways (5). Depending on the stage of the cell cycle, the checkpoints lead to different outcomes. The G1/S checkpoint is induced by DNA damage and arrests the cell cycle before DNA synthesis starts. The protein p53, a tumor suppressor, plays an important role. In healthy cells p53 is instable, but if DNA damage occurs, this protein gets stabilized and activates further proteins involved in cell cycle control and DNA repair (6). In case of severe DNA damage, it activates pro-apoptotic proteins, which lead to controlled cell death, called apoptosis (Figure 1) (3, 7). Damage during replication can lead to a stalled replication fork. The intra-S checkpoint ensures, that it gets stabilized (Figure 1), thereby preventing the replication fork from an irreversible collapse (3). The G2/M checkpoint controls that the chromosomes entering mitosis are intact and can be separated in a correct manner (Figure 1). This is a very important checkpoint, because damaged chromosomes can lead to mistakes in separation or

DNA strand breaks. This can cause chromosomal aberrations or even aneuploidy and have severe consequences for the cell (3, 8).

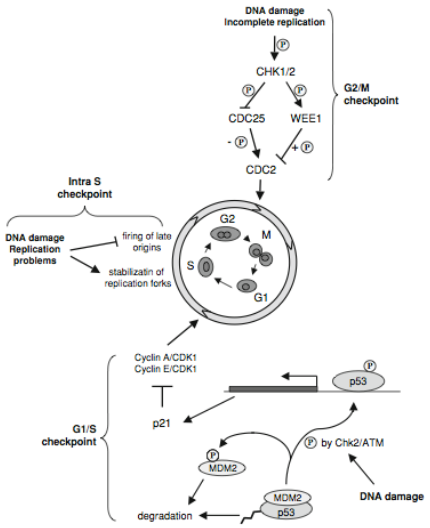

Figure 1: Cell cycle proteins and different cell cycle checkpoints. *Schematic representation of cell cycle proteins and processes targeted by different DNA damage checkpoints. DNA damage can arrest cell cycle progression at the G1/S and G2/M transitions, and slow down S-phase progression. A main target of the G1/S checkpoint is the tumor suppressor protein p53. In undamaged cells, p53 forms a complex with the ubiquitin ligase MDM2. The constitutive ubiquitylation of p53 targets it for proteosomal degradation, thus ensuring a rapid turnover of p53. DNA damage activates signalling cascades, which act to stabilize and activate p53 via multiple redundant mechanisms, including phosphorylation of p53 by CHK2 and ATM, and phosphorylation of MDM2 (9). Stabilized p53 activates transcription of many genes including the gene encoding p21, an inhibitor of cyclin-dependent kinases (CDKs). Increase in p21 expression suppresses cyclin E and cyclin A-associated CDK activities, which are necessary for entering the S phase. The S-phase checkpoint is activated by replication problems, promotes stabilization of replication forks and inhibition of late origin firing. The G2/M checkpoint prevents cell division in the presence of damaged or unreplicated DNA. The main cell cycle protein targeted by this checkpoint is CDC2, which controls entry into mitosis. CDC2, on the other hand, is negatively regulated by phosphorylation by the WEE1 kinase and positively regulated by de-phosphorylation by the CDC25 phosphatase. In response to DNA damage checkpoint, proteins CHK1 and/or CHK2 phosphorylate both WEE1 and CDC25. Phosphorylation activates WEE1, which in turn phosphorylates CDC2 and this results in inhibition of CDC2 and therefore prevents cell division. CDC25 is inhibited by phosphorylation and is no longer able to de-phosphorylate and thus can no longer activate CDC2. Cells exposed to different of DNA damaging agents activate DNA damage response (DDR). It consists of cell cycle arrest which is followed by DNA repair or, if the damage is too big to be repaired, induction of apoptosis or senescence. For the coordination of the DDR, the interplay between different posttranslational modifications (PTM) like ubiquitylation, phosphorylation, acetylation or sumoylation is very important (10). These PTM are attached to their substrate proteins by a variety of different enzymes. (reproduced from reference (3)).*

The enzymes responsible for phosphorylation are protein kinases, which transfer phosphate groups from ATP to a substrate protein. Important kinases for the checkpoint controls are the ATM/ATR kinases, which recruit repair factors to the damaged DNA and facilitate the DNA damage repair by transcriptional or posttranscriptional induction of repair proteins (2). When the damage cannot be repaired, cell death by apoptosis is the last way out. Apoptosis is an important function for the organism, because this prevents that cells accumulate mutations and therefore might undergo transformation. Therefore apoptosis is a mechanism which prevents tumorigenesis (2).
DNA synthesis is carried out during the S-phase of the cell cycle. This is accomplished by the replicative DNA polymerases (Pols), which are extremely precise and efficient in copying DNA. On one hand it is important, that the information encoded in the DNA is not modified, but on the other hand the replicative Pols have problems to use a DNA template that is damaged. As a consequence, replication is slowed down or completely stalled by most of the DNA lesions (3). Despite the fact that DNA has to be kept very stable, it has been shown that about 100,000 lesions are generated per day in the genome of every single cell (11). This is because DNA is exposed to many damaging agents, not only exogenous influences, like UV light, ionizing radiation or toxic chemicals, but also endogenous influences, like reactive oxygen species or free radicals produced upon oxidative phosphorylation in the mitochondria. Reactive oxygen species and free radicals are side products of the normal metabolism and therefore not avoidable (3, 12).

3.2 The five DNA repair mechanisms

Pols are enzymes that synthesize a new DNA strand complementary to a template DNA and act in a unidirectional process to synthesize the new DNA in a 5' to 3' direction. Due to this directionality, and the fact that both of the DNA strands are replicated simultaneously, replication differs slightly between the two DNA strands. The leading strand can be synthesized in a continuous manner, mainly by Pol ε, while the lagging strand has to be synthesized discontinuously. Replication of the lagging strand is mediated mainly by Pol δ, which replicates short fragments of about 200 base pairs length, called Okazaki fragments. As Pols δ and ε can only extend from existing DNA primers, DNA replication has to be initiated by the Pol α/primase complex, which produces a short fragment of RNA, followed by DNA that can subsequently be used by the other Pols to replicate the DNA. Replication takes place during each cell cycle. Even though replication is a highly faithful process, mistakes can occur occasionally during replication.

To repair, on the other hand, the thousands of damages in DNA many repair mechanisms have evolved. The different DNA repair mechanisms can be categorized into 5 different sub-pathways: i) non-homologous end-joining (NHEJ), ii) homologous recombination (HR), iii) mismatch repair, iv) nucleotide-excision repair and v) base excision repair (BER) (Figure 2). While the first two deal with DNA double-strand breaks, the last three repair damages, that do not lead to double-strand breaks. In what follows are the functions of Pols discussed in the frame of these five DNA repair pathways.

(i) NHEJ repairs double-strand DNA breaks by ligating two ends. Since the homologous DNA template is not needed for the repair, this pathway is considered to be error prone (2, 13). This pathway is independent of a DNA template and it can take place in any phase of the cell cycle. NHEJ plays an important role not only in the repair of damage-induced DNA breaks, but also in the V(D)J recombination during maturation of pre B cells (13). Pols λ, μ and TdT were found to be important in this repair pathway (15).
(ii) The second pathway dealing with DNA double strand breaks is HR. In contrast to the NHEJ, the HR uses a template DNA to repair breaks as accurately as possible, and is therefore considered to be error free (13). Due to the need of a homologous sequence for HR, it can only take place in late-S or G2 phase, when the sister chromatid can serve as a template (2). Several Pols participate in this process, such as Pols δ, ε, ζ, λ, μ, and Tdt (14-19).
(iii) The MMR pathway is responsible for the restoring mismatches and gaps, which occur after DNA replication (Figure 2). In this pathway, the mispaired segment of the daughter strand is excised and removed. This gives second chance to Pol δ/e holoenzyme, composed of either Pol δ or Pol ε, replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA) for correct incorporation (13).
(iv) The NER pathway (Figure 2) is responsible for repair of lesions that cause distortions on one DNA strand. Such distortions are recognized by specific proteins unwinding the DNA to ensure that specific endonucleases can remove oligonucleotides of 22-30 bases. Subsequently, Pols δ, ε, κ or η are recruited to the DNA to fill in the gaps (2, 13, 20-23).
(iv) The BER pathway is responsible for repairing bases that have been damaged by depurinations, depyrimidations, oxidations or deaminations. These damaged bases are recognized by specific DNA glycosylases, which excise the damaged or a mispaired base to pave the way for further processing by other enzymes. Two sub-pathways of BER exist (24). One is called short-patch (SP) BER and is used if just one nucleotide has to be incorporated. The second, called long-patch (LP) BER, is defined as pathway that inserts 2-12 new nucleotides (25). Several Pols are able to work in the BER repair pathway, such as Pols β, λ, θ, δ, and ε (26-29). For the master thesis presented here, Pol β is the most relevant enzyme.

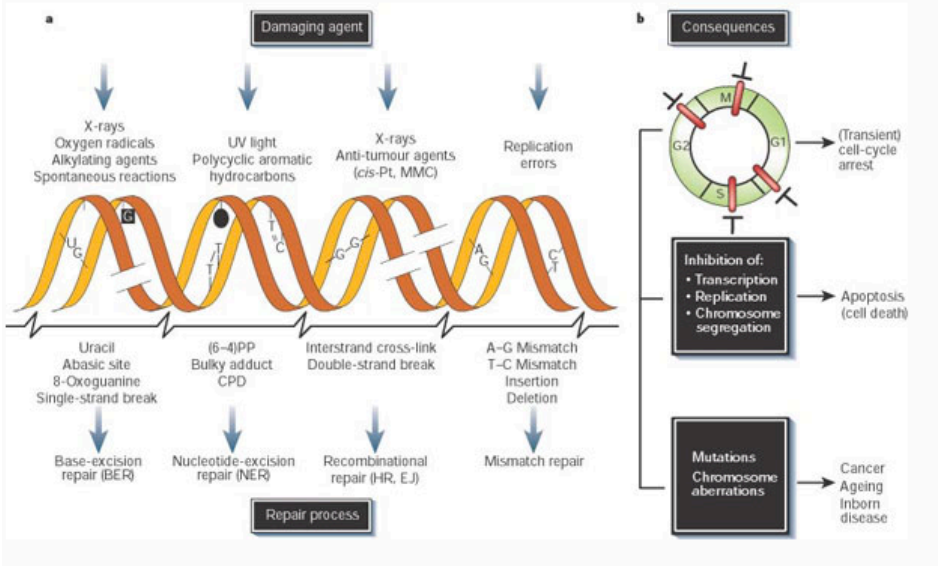

Figure 2: DNA damage, repair mechanisms and consequences *(from reference (30)) For details see text.*

3.3 Base excision repair (BER)

DNA damages induced by oxidation, alkylation and non-enzymatic methylation lead to small base lesions, which are repaired by BER (12). There are two different sub-pathways of BER, namely SP-BER and LP-BER, dependent on the number of nucleotides that have to be incorporated. Figure 3 summarizes the different pathways of BER. Most lesions are repaired via SP-BER, this pathway is a Pol β-dependent pathway (31, 32). Mono- or bifunctional glycosylases remove the damaged nucleotide, which leads to apurinic/apyrimidinic site (AP site) with a 3`OH and a 5`deoxyribose-phosphate moiety (5`dRP) (32). Pol β has polymerase and dRP lyase activities, therefore being able to hydrolyse the 5`dRP moiety and refill afterwards the single nucleotide gap (31). The last step is the ligation, carried out by a complex of DNA ligase IIIα (lig) and XRCC1 (31).
The initiation of the LP-BER is similar to the initiation of the SP-BER leading to a nicked DNA. In case the AP site is reduced or oxidised, the 5`end lyase activity of Pol β is not able to remove the altered AP site (33). That results in a switch to the LP-BER that can be subdivided in two distinct sub-pathways. The first one is a PCNA dependent sub-pathway, where Pol β incorporates the first nucleotide and then the replicative Pols δ and ε are recruited by PCNA to continue the synthesis of the new strand (34). The second pathway is called “hit and run”, because flap endonuclease 1 (FEN-1) excises the damaged DNA strand and Pol β continuously re-synthesises the new strand (24, 35-37).

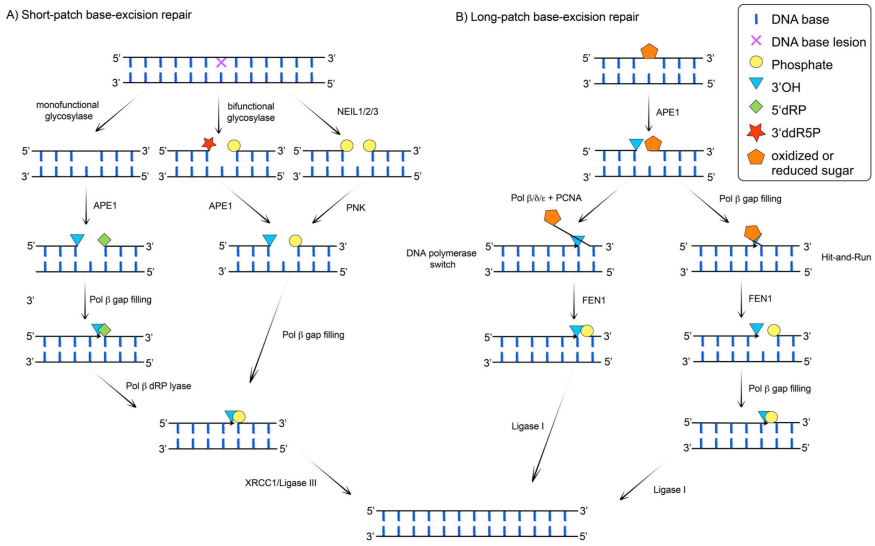

Figure 3: Scheme of short-patch and long-patch base excision repair (SP-BER and LP-BER) sub-pathways*. PNK: polynucleotide kinase (reproduced from reference (38)) For details see text.*

3.4 DNA polymerases

Pols play an important role in many DNA transactions such as in DNA replication, DNA repair, in translesion DNA synthesis, cell cycle control and DNA damage checkpoint (39). They are in addition involved in immunoglobulin recombination, generation of antigen receptors and in replication and repair of mitochondrial DNA (40, 41). Pols are classified in seven different polymerase families, according to their sequence homology and structural similarities (42). In eukaryotes the Pol families A (γ, θ, υ), B (α, δ, ε, ζ), X (b, λ, μ, TdT) and Y (η, ι, ϰ, Rev1) were identified (11, 42). The Pols of the B family are responsible for DNA replication, whereas the specific function of the X family Pols consists in gap filling during DNA repair (11). The Y family Pols are able to synthesize over many DNA lesions, but they have therefore a higher error rate than replicative Pols (13).
Due to differences in structure, the fidelity of Pols reaches from 10^{-1} to 10^{-6} and results in variety of functions the Pols have to fulfil (12). There are highly conserved domains within the different families of Pols. All Pols contain three different domains, which resemble a right human hand. These domains are called palm, thumb and fingers (42). The fingers and thumbs show differences between the different Pol families, while the palm domain is highly conserved. It is known, that this domain contains the active site of the Pols (43). Most of the Pols need a DNA template to which a primer with 3'-OH is annealed and a divalent cation, like magnesium or manganese, has to be present (13). Often Pol auxiliary proteins are required such as PCNA (44), RF-C and replication protein A (RP-A) (45-47).

Pols always synthesize DNA in the 5' to 3' direction but some Pols, like Pol δ and Pol ε, in addition have a 3'-> 5' proofreading activity that increases the fidelity of DNA replication (48, 49). The Pol reaction starts with a hydrogen bonding between the correct dNTP and the DNA template. This removes water from the active site and makes the geometric selection on the active site possible. Due to the dNTP binding affinity of the Pol, a conformational change in the active site takes place, whereby a phosphodiester bond between the primer and the dNTP is established, which releases a pyrophosphate (13, 49, 50).

3.4.1 DNA polymerase β

Pol β belongs to the X family Pols and has to fulfil different functions in vertebrates, like DNA repair, V(D)J recombination, as well as translesion synthesis (42). Pol β is a single 39 kDa polypeptide of 335 amino acids (51) and contains two different domains. One is located near the C terminus and contains the Pol activity, while the second domain is located near the N terminus and is responsible for the dRP-lyase activity and the binding to the single-stranded DNA (52-55). The Pol X family members are mostly involved in DNA repair and especially in the filling of small gaps from one to a few nucleotides (11, 56). In mammals Pol β is a highly conserved Pol, but it is not present in bacteria, plants and protozoa (57). It was shown, that Pol β prefers gapped DNA as a template, which contains a 5' phosphate on the downstream strand. If this downstream strand is missing or the gap is to big, the Pol β binding affinity is drastically reduced (58). Important for Pol β binding to the DNA is the downstream 5' phosphate (13). This explains, why the dRP-lyase activity of Pol β is

much more important for preventing the cell from cytotoxic DNA damage, than the polymerase activity itself (59). Pol β plays a major role in SP-BER (42), where both, the dRP-lyase activity and the Pol activity are essential (60). Given that Pol β is expressed in mouse and rat testis, it seems likely that Pol β also plays a role in mammalian meiosis (61). As mice carrying a targeted disruption in the Pol β gene suffer from growth retardation and death after birth, due to respiratory failure, it can be concluded, that Pol β is also necessary for neural development (62). A complete loss of Pol β is not compatible with life (63).
It is furthermore important that Pol β levels are tightly regulated. In some tumors Pol β is overexpressed, leading to the conclusion that Pol β up-regulation might cause genomic instability and onset of cancer (64). Increased Pol β transcription in cells exposed to oxidative stress can lead to a mutator phenotype (65). Finally, cells with increased Pol β levels are much more sensitive to treatment with radiation (66).

3.5 Interaction partners of DNA polymerase β

3.5.1 X-ray repair cross complementing protein 1

The X-ray repair cross complementing protein 1 (XRCC1) is a 633 amino acid protein of approximately 70 kDa (67). XRCC1 is a scaffold protein, which forms large multiprotein complexes with several DNA repair proteins, like DNA glycosylases, AP endonuclease-1 (APE-1), poly (ADP-ribose) polymerase 1 (PARP-1), PARP-2, poly nucleotide kinase (PNK), Pol β, lig III and PCNA (68-77). The binding sites for Pol β and lig III were determined and shown to contain overlapping binding epitopes (67). XRCC1 itself does not have any, so far identified, enzymatic activity, but the complexes XRCC1 forms are involved in single-strand break repair (SSBR) and BER (78). SSBR is defined as the repair of single-strand breaks in DNA caused by irradiation, incomplete topoisomerase activity or ROS by-products (31).
The N-terminal domain of XRCC1, from amino acid 1 - 183, was shown to have a high affinity to Pol β (75). The complex containing Pol β and XRCC1 encircles gapped DNA (75, 79). Binding of XRCC1 to Pol β stabilizes on the one hand side the protein by preventing its degradation and acts on the other hand side as a recruiting factor for Pol β that targets the protein to damaged DNA (78, 80). The binding affinity increases by an order of magnitude when XRCC1 is oxidized and simultaneously the oxidation prevents a direct interaction of XRCC1 with the damaged DNA (81).

3.5.2 Proliferating cell nuclear antigen (PCNA)

PCNA, the *E.coli* DNA pol III β-subunit and the bacteriophage T4 gene45 protein belong to the DNA sliding clamp family (β clamps). These proteins fulfil important functions, what might be the reason why they are highly functionally conserved (82). PCNA is a ring-shaped trimetric complex, each homotrimer is formed of three monomers, that contain two identical domains. The ring-like structure enables PCNA to encircle the DNA and to be part of the replisome (83, 84). PCNA is involved in many different cellular pathways like Okazaki fragment processing, DNA repair (BER, NER, MMR), translesion DNA synthesis, DNA methylation, chromatin remodelling and cell cycle regulation, as well as in apoptosis and transcription (44, 85,

86). Posttranslational modifications of PCNA, like ubiquitylation, are important for DNA damage repair to take place and to maintain the genomic integrity (87, 88). Even though the current knowledge suggests, that PCNA is not directly regulated by kinases like ataxia-teliangiectasia mutated (ATM) or ataxia-telangiectasia and Rad3-related protein (ATR), which are induced through DNA damage (89).
Figure 4 documents important interaction partners of PCNA, which are needed to modulate the functions PCNA has in the metabolism of mammalian cells (90). Most of the so far identified interaction partner carry the PCNA interacting protein (PIP) box (90).

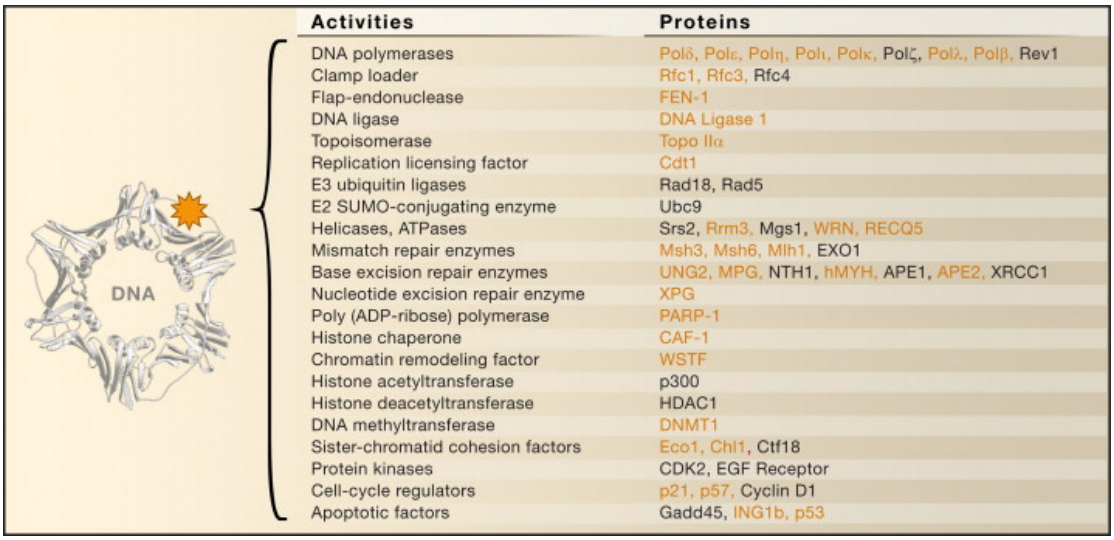

Figure 4: PCNA-binding proteins. *PCNA interacts directly with many proteins, which are involved in many different cellular processes. Above a selection of key PCNA-dependent activities and the corresponding PCNA-interacting proteins is listed. Proteins in orange are known to contain PIP-box sequences, which bind to PCNA (orange) (reproduced from reference (90)).*

PCNA was shown to interact with different replicative Pols thereby enhancing their processivity by binding the Pols to the DNA and preventing their dissociation from the DNA (13, 91). Additionally, PCNA was identified to bind to Pols involved in the BER like Pol ε, Pol δ and Pol β (33, 91-93). Kedar et.al. identified three regions in Pol β, that resemble the PIP box and are able to bind to PCNA *in vivo* and *in vitro* (93). Furthermore it was found, that PCNA enhances Pol β activity in the LP-BER by stimulating FEN-1, whereas no improvement of the Pol β activity in SP-BER was observed (33). Posttranslational modifications of Pol β are known to have an effect on the binding capacity to PCNA. El-Andaloussi et al. from our laboratory previously showed, that methylation of Pol β by protein arginine methyltransferase 1 (PRMT1) prevents binding of PCNA to Pol β and therefore Pol β is no longer able to fulfil its function (94). Further information about the effects of posttranslational modifications are rare, underlining the importance of this investigation, examining which effect the phosphorylation of Pol β has on the interaction with PCNA.

3.6 DNA polymerase β, XRCC1 and PCNA in BER

Upon application of low doses of DNA damaging agents to cells XRCC1 co-localizes with Pol β and PNK to the sites of DNA damage. Higher doses of damaging agents, like irradiation, provoke a recruitment of PCNA and FEN-1 to the damaged DNA. In this pathway XRCC1 plays a major role, by enhancing the recruitment to the damaged DNA (33, 78). The interaction of Pol β with XRCC1 is of great importance for BER. It was shown, that mutated XRCC1/ligIIIα complexes, which can not interact with Pol β, are unable to restore damaged DNA (95). Additionally the absence of Pol β in cell extracts decreases the recruitment of the XRCC1/ligIIIα complex to sites of DNA damage two-fold (95, 96). The other way around, Lan et.al. showed that in XRCC1-defective EM9 cells, Pol β was not able to repair UVA-induced DNA single strand breaks (SSB) indicating that the repair of UVA-induced lesions is XRCC1-dependent (97).
Moreover, the interaction of Pol β with PCNA plays an important role in BER since PCNA improves the capacity of Pol β in LP-BER (33). XRCC1 binds to PCNA even though XRCC1 lacks a PCNA interaction protein (PIP) like a PIP box or AlkB homologue 2 PCNA-interacting motif (98). Purified, recombinant PCNA was shown to bind to purified, recombinant XRCC1 between amino acid 166 - 310 (77). Fan and co-workers reported, that XRCC1 and PCNA interact in undamaged HeLa cells only in S-phase of the cell cycle at sites of DNA replication (77).

3.7 Regulation of DNA polymerase β by postranslational modifications

The BER pathway has to be tightly regulated to ensure a correct repair of damaged DNA. A possibility to do so is to control the steady-state levels or the activity of the involved enzymes by posttranslational modifications. Ubiquitylation was shown to play a role in regulation of Pol β, XRCC1 and lig III. The E3 ligase Mule is located in the cytoplasm and is responsible for monoubiquitylation of Pol β (99). Monoubiquitylated Pol β is polyubiquitylated by the major E3 ubiquitin ligase carboxyl terminus of Hsc70 interacting protein (CHIP), which thereby leads to its proteasomal degradation (80). Non-ubiquitylated Pol β is located to the nucleus, to sites of DNA damage at the chromatin (Figure 5). Consistently, in Mule depleted cells Pol β levels are increased (100).
Another protein involved in the regulation of Pol β is ARF. ARF is normally located in the nucleolus, but in case of DNA damage, it is released and can interact with and inhibit Mule, what therefore prevents the ubiquitylation of Pol β (101). In this case more Pol β is available to enter the nucleus and act on the sites of DNA damage (Figure 5) (100, 102). This pathway was shown to be responsible for regulation of 15-20% of the cellular Pol β only. Therefore it implies, that ubiquitylation is not regulating Pol β levels in case of severe exogenous DNA damage, but is rather responsible for maintaining a constant steady state level (100).

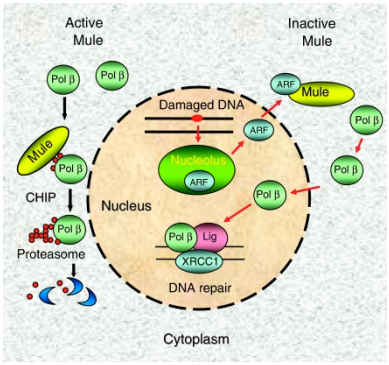

Figure 5: Proposed model of the regulation of DNA polymerase β steady-state levels by Mule, ARF and CHIP. *If not engaged in DNA repair Pol β is ubiquitylated by Mule that is then a target for CHIP mediated polyubiquitylation and subsequent degradation by the proteasome (left side of the scheme). However, after detection of DNA damage ARF is released from the nucleolus into the cytoplasm in which it inhibits the activity of Mule, thus reducing Pol β degradation and up-regulating DNA repair (right side of scheme). The repair of DNA damage will result in a decreased release of ARF, with a concomitant increased activity of Mule that will down-regulate Pol β levels. A new adjustment cycle will therefore begin on the detection of increased levels of DNA damage. For more details see text (reproduced from reference (100)).*

Another posttranslational modification of Pol β is acetylation. Pol β becomes acetylated through the transcriptional co-activator p300. In case of acetylation the dRP-lyase activity of Pol β is reduced, while the Pol activity is not affected. As consequence of the reduced dRP-lyase activity the BER pathway is affected (103-105). Methylation of Pol β by protein arginine methyltransferase 6 (PRTM6) stimulates the Pol activity, whereas the dRP-lyase activity is not affected (106). Therefore it was suggested, that PRTMs play a role in DNA damage signalling (107). Finally, Kotake et al. showed, that the activity of Pol β extracted from rat adrenal glands depends on the phosphorylation level of Pol β (108). These authors tested the dependence of Pol β from cAMP dependent protein kinase (A-kinase) and found that this kinase increased the activity of Pol β. Finally, Tokui and co-workers showed, that the phosphorylation of Pol β by PKC leads to its inactivation (109). In contrast to the above mentioned findings, we found that phosphorylation of Pol β WT by PKC does not affect its activity on a single nucleotide gap primer/template (1).

3.8 Recent developments according DNA polymerase β - update since 20.12.2012

As mentioned above, Pol β is the main enzyme in BER, but the synthesis reaction of Pol β is yet poorly understood on the atomic level. Recent result propose, that a carboxylate group of Asp256 may be responsible for the synthesis reaction, by accepting a proton from the O3`group. It was found, that the synthesis activity of a Pol β mutant (D256E) in which Asp256 was changed to Glu is decreased 1000-fold. BER and MMR play an important role in the cytotoxicity of the chemotherapeutic cis-Diamminedichloroplatinum (II) (cisplatin). It was found, that cells defective in BER and MMR showed a cisplatin-resistant phenotype. For characterization of the effect, a mutant of Pol β was used (D256A) that is deficient in polymerase activity (110).

In recent studies on the structure of Pol β it was observed, that Pol β has not just two metal binding sites, but there exists a third transient metal binding site, which was only observed in case of a correct nucleotide insertion (111).
1000 to 7000 oxidative DNA damages occur per day per cell and Pol λ was shown to be the key Pol in repairing these lesions. DNA Pol δ-interacting protein (PolDIP2) was found to support Pol λ in its function repairing the 8-oxo-7,8-dihydroguanine damage. Further it was investigated, whether PolDIP2 has the same influence on other Pols, like Pol β. It was shown that PolDIP2 does not influence Pol β, by enhancing Pol β activity in repairing the damaged DNA caused by ROS (112).
Pol β is also known to play a role in carcinogenesis. Zhao et al. found, that Pol β overexpression leads to a associated genetic instability and may therefore play a key role in Benzo[a]pyrene carcinogenesis (113). Another pathway of tumorigenesis was identified in esophageal squamous cell carcinoma, where a mutation of the Pol β promoter was observed, which mostly leads to a C --> A mutation at locus - 37 (114).
It was shown, that Pol β also is the main factor in DNA repair in macrophages and a connection of Pol β in repair mechanisms in macrophages and the insertion of the proviral HIV-1 DNA was identified. It is known, that the integration of HIV-1 proviral DNA in macrophages is delayed, Van Cor-Hosmer et.al. suggested that this is due to the low levels of dNTPs available in macrophages (115).

3.9 Protein kinase C (PKC)

The family PKC's comprises ten different isoforms of serine/threonine kinases (116). The function of these kinases is to catalyze the covalent transfer of phosphate moieties from ATP to serine, threonine or tyrosine residues on their protein substrates. The different PKC isoforms all consist of a single polypetide chain (117).

3.9.1 Regulation and structure of protein kinase C

Kinases phosphorylate several important proteins, therefore they have to be tightly regulated. The N terminal region contains a regulatory function, whereas the C terminal region contains the kinase domain (117, 118). The inactive form of PKC is located in the cytosol, where its pseudosubstrate, an autoinhibitory domain is bound to the kinase domain forming a hairpin-like structure. PKC is activated by the lipid second messenger diacylglycerol (119), by Ca2+ and by phosphatidylserine (120). Upon activation, PKC is recruited to the membrane, that enables the abrogation of the binding between the pseudosubstrate and the kinase domain, the activation loop of PKC gets exposed and thus can be phosphorylated (117).
PKC itself is phosphorylated by the protein kinase PKD-1 in two steps. First, the T-loop of the PKC kinase domain is phosphorylated, and second, the turn motif becomes phosphorylated, whereby PKC is changing into a thermally stable closed state (121, 122). The third phosphorylation needed to activate PKC is an autophosphorylation triggered by the first phosphorylation step. Presumably, this auto-phosphorylation manages the subcellular localization of PKC (119).

3.9.2 Signaling

PKC is known to phosphorylate different molecules and play a role in activation of these substrates. Among the DNA repair proteins, it is known that PKC interacts and

phosphorylates the chromatin bound OGG1 DNA glycosylase, but the function of this phosphorylation is so far unknown (123). The phosphorylation of the MUTYH DNA glycosylase by PKC has been shown to have an activating effect on MUTYH (124).
cAMP response element binding protein (CREB) is a transcription factor with a weight of 43-kDa, that is composed of three domains, a C-terminal DNA-binding domain, a leucine zipper dimerization domain and a transcriptonal activation domain (125). CREB activation via phosphorylation is an important step for numerous signal transduction pathways (126). The CREB-induced gene transcription requires phosphorylation on Ser 133 (127, 128). As CREB is a downstream target of PKC, PKC is able to phosphorylate CREB on Ser 133 and therefore activate it (125). Different factors are known, for modulating the PKC-dependent phosphorylation pathway. For example the phosphorylation can be simulated upon treatment with PMA or the other way around, the phosphorylation can be blocked upon treatment with bisindolymaleimide I (125).

3.10 Summary of the master thesis

In the master thesis it was shown, that human DNA Pol β is phosphorylated by PKC in the N-terminal dRP-lyase domain (1). After identification of possible phosphorylation sites in Pol β, these sites were mutated by site-directed mutagenesis. The point mutants T10A, S30A, S44A, S55A, T67A and S180A were tested to investigate whether PKC is able to phosphorylate them. The results of the *in vitro* phosphorylation assay showed clearly, that all of the point mutants of Pol β are less phosphorylated, than the Pol β WT leading to the conclusion that these sites are important for phosphorylation of Pol β. Furthermore, the activity of these mutants was tested. Therefore the phosphoablated Pol β mutants were tested on a *17/73 mer primer/template and a *39/100 mer primer/template and the result was compared to the activity that Pol β WT has under these conditions. It was observed, that there are not only differences in the activity of the different mutants itself, but also in the efficacy of Pol β WT , depending on what primer/template condition was used. This suggested, that the activity of Pol β is primer/template dependent. In another experiment the differences between the activity of phosphorylated Pol β WT and non-phosphorylated Pol β WT were investigated. Therefore the activity on a one nucleotide gap, which represents the physiological substrate of Pol β in SP-BER, was evaluated. No differences in the activity of phosphorylated Pol β WT compared to the non-phosphorylated Pol β WT could be detected.
Taken together, the results shown in the master thesis suggested that the activity of Pol β is not altered significantly upon phosphorylation by PKC (1). This prompts the question whether the phosphorylation by PKC is important for the activity of Pol β. PTMs in general can also have important roles in the subcellular localization and the stability of the modified protein. Therefore, it would be important to determine in further studies, whether the stability of Pol β changes upon activation of PKC signalling. Along this line, it would be interesting to analyse whether changes can be observed in the association of Pol β with its interaction partners upon phosphorylation.

4 Aim of the thesis

After it was shown *in vitro*, that phosphorylation of Pol β by PKC has no influence on its activity, the aim of this thesis was to study what effect the phosphorylation by PKC has on the stability of Pol β and on the interaction of Pol β with the interaction partners XRCC1 and PCNA.

5 Material und Methods

5.1 Materials

5.1.1 Buffers

SDS PAGE
4x SDS upper gel
0.5 M Tris-HCl, pH 6.8
0.4% SDS

4x SDS lower buffer
1.5 M Tris-HCl, pH 8.8
0.4% SDS

TBS
10 mM Tris-HCl, pH 8
50 mM NaCl

TBS-T
10 mM Tris-HCl, pH 8
50 mM NaCl
0.1% (v/v) Tween

10x Lämmli buffer
600 mM Tris-HCl, pH 6.8
20% SDS
20% Glycerol
0.05% Bromphenol blue
20% β - mercaptoethanol

5 x Running buffer
0.125 M Tris base
0.96 M Glycine
0.5 % SDS

PBS 1x
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.76 mM KH_2PO_4
pH 7.4

Pulldown Buffer
40 mM Tris, pH 7.5
100 mM NaCl
0.1% NP-40
5 mM Imidazol
1 μg/ml Pepstatin

1 µg/ml Leupetin
1 µg/ml Bestatin
1 mM PMSF
1 mM NaF
5 mM $Na_4P_2O_7$
10 mM Glycerol-Phosphate

Lysis Buffer
10% Glycerol
1% Triton X-100
1.5 mM $MgCl_2$
50 mM Hepes pH 7.5
150 mM NaCl
1 mM EGTA
100 mM NaF
10 mM $Na_4P_2O_7$
1 µg/ml Pepstatin
1 µg/ml Leupetin
1 µg/ml Bestatin
1 mM PMSF

10 x Transfer Buffer
25 mM Tris
200 mM Glycine
20% (v/v) MeOH

BBS 2x
50 mM BES
280 mM NaCl
1.5 mM Na_2HPO_4 x $2H_2O$
pH 6.96

HNTG Buffer
20 mM Hepes pH 7.5
10 mM NaF
150 mM NaCl
0.1% Triton X-100
10% Glycerol

5.1.2 DNA polymerase β point mutants

DNA pol β point mutants (T10A, S30A, S44A, S55A, T67A, S180A) were generated according to the protocol which is described in the master thesis "*Phosphorylation of Human DNA Polymerase β by Protein Kinase C*" (1)

5.1.3 Antibodies

Antibody	Concentration used	Company
H3	1:1000 rabbit	abcam
Phospho-CREB	1:1000 rabbit	Cell Signaling
Phospho-PKC	1:1000 mouse	Cell Signaling
Flag	1:1000 rabbit	Sigma
HA	1:1000 mouse	Covance
c-myc	1:1000 mouse	Roche
hPolβ - His 6x	(0.23mg/ml) rabbit No.42	Purified in the laboratory
Pol β	1:500	Thermo
Pol β	1:1000 mouse	abcam
Tubulin	1:10 000 mouse	Sigma
mouse 2nd AB	1:15 000	Li-Cor
rabbit 2nd AB	1:15 000	Li-Cor

5.1.4 Cell lines

5.1.4.1 HEK293T

Human embryonic kidney cells (American Tissue Culture Collection) were cultivated in DMEM high glucose (4.5g/l) with stable Glutamine medium (PAA Laboratories GMbH), 10% FCS (Gibco) and 1% Penicillin-streptomycin (10 000 U/ml, Gibco).

5.1.4.2 HeLa

Human HeLa cells, cervical cancer cells (American Tissue Culture Collection), were cultivated in DMEM high glucose (4.5g/l) with stable Glutamine medium (PAA Laboratories GMbH), 10% FCS (Gibco) and 1% Penicillin-streptomycin (10 000 U/ml, Gibco).

5.1.4.3 T24

The T24 human urinary bladder cancer cells were cultivated in DMEM high glucose (4.5g/l) with stable Glutamine medium (PAA Laboratories GMbH), 10% FCS (Gibco) and 1% Penicillin-streptomycin (10 000 U/ml, Gibco).

5.1.5 PMA (Sigma)

Phorbol-12-myristat-13acetat is a structural analogue to diacylglycerine and is used to activate protein kinase C.

5.1.6 ro 32-0432 (Sigma)

Bisindolylmaleimide XI hydrochloride is a selective inhibitor of protein kinase C.

5.2 Methods

5.2.1 Western blot analysis

The proteins were separated on a SDS gel (10% Acrylamid 37.5:1), 30 minutes at 80V and in the following 1.5h at 120V with the BioRad blotting apparatus. They were transferred at 4°C on a PVDF FL membrane (Imobilon) for 50 minutes at 100V. The membranes were blocked with blocking buffer (Odyssey Blocking Buffer Li-Cor, dilution 1:2 in PBS) for 30 minutes at room temperature. The first antibodies were added and incubated overnight. The membranes were washed 3 times for 5 minutes with TBS-T and one time with TBS. The corresponding secondary antibodies were added and incubated for 1 hour in darkness. Membranes were again washed 3 times with TBS-T and one time with TBS and analysed using the "Odyssey Infrarot Scanner" Li-Cor).

5.2.2 HIS-pulldown

For the HIS-Pulldown the lysates of transfected HEK293T cells were prepared on ice, using pre-cooled buffers. The 10 cm plates were washed 2 times with 5 ml ice cold PBS. 1 ml ice cold PBS was added to the plates, the cells were scratched in an Eppendorff tube, centrifuged at 5000 rpm for 5 minutes. For re-suspending the pellet 1 ml of the lysis buffer was added and the cells were vortexed and left on ice for 3 minutes. To break the cells they were sonicated (BioRuptor, Diagenode) 2 times for 30 seconds. Afterwards the samples were centrifuged at 12 000 rpm for 10 minutes. The pellet was discarded and the protein concentration in the supernatant was determined using the Bradford reagent (BioRad). For the pulldown, the Ni-beads (ProBond™ Nickel-Chelating Resin, Invitrogen, 50μl/sample) were coated overnight with pulldown buffer and 10% BSA (Fluka, 10 x vol. of the beads). Afterwards the beads were washed 3 times with 1 ml pulldown Buffer, in between the samples were rotated for 5 minutes and centrifuged at 2000 rpm for 1 minute. The beads were re-suspended in pulldown buffer with a final volume of 500 μl. 1 μg of Pol β was added to the beads, the samples were rotated for 1 hour at 4°C. The beads were washed 3 times with 500 μl pulldown buffer. In between the samples were rotated 5 min at 4C° and then centrifuged at 2000 rpm for 1 minute. 500 μg of lysate was added to the beads and the samples were again rotated for 2 hours at 4°C. After rotating the beads were washed 3 times as described above. After washing 30 μl 2x Lämmli Buffer was added to the beads and the samples were boiled for 10 minutes. For the input 10% of the used lysate and 0.5μg Pol β were loaded.

5.2.3 Calcium-phosphate transfection

$3.6x10^6$ HEK293T cells were seeded in a 10 cm dish, cells were cultivated overnight with DMEM medium as described in 5.1.4.1 at 37°C and 5% CO_2. After 12 hours the cells were transfected with pET-21 b (+)-Pol β and pcDNA3-HA-XRCC1 or pET-21 b (+)-Pol β and pcDNA3-N-myc-PCNA plasmids, respectively. H_2O (550 μl), $CaCl_2$ (64μl, 2.5 M), BBS (640 μl), and 8 μg DNA were mixed and vortexed for 10 seconds. The mixture was added to the medium. 12 hours after the transfection the medium was exchanged and the transfected HEK293T cells were again incubated overnight before harvest.

5.2.4 Co-immunoprecipitation

12 hours after transfection the medium was exchanged and the cells were returned to the incubator overnight. For activation or inhibition of PKC respectively, the cells were treated with 200 nM PMA or with 200 nM PMA+100 nM ro 32-0432 for 1 hour, both components were solved in DMSO (Sigma-Aldrich) whereby a control dish was treated with DMSO alone.
Lysate (all on ice, cooled centrifuge): The plates were washed with 5 ml cold PBS and the cells were flushed off the plate with 1 ml PBS into an Eppendorff tube. Afterwards the cells were centrifuged at 5000 rpm for 5 minutes at 4°C. The pellets were vigorously re-suspended in 500 μl of lysis buffer and left on ice for 6 minutes. Again the samples were centrifuged at 12 000 rpm for 10 minutes at 4°C, the pellet was discarded and 5% of the supernatant was loaded as input. The same volume HNTG buffer was added to the remaining supernatant. Further the Flag-antibody (2μl) was added and the sample was rotated for 2 hours at 4°C. The ProtG Sepharose beads (25 μl, GE Healthcare) were 2 times washed with 1 ml HNTG buffer. Between the washing steps the beads were centrifuged at 5000 rpm for 1 minute. Afterwards the beads were added to the samples and rotated for 1 hour at 4°C. Finally the samples were washed 3 times with 500 μl HNTG buffer. In between the washing steps the samples were rotated for 5 minutes and then centrifuged at 5000 rpm for 1 minute. To prepare the samples for the SDS-PAGE 30 μl 2x Lämmli Buffer was added to the pellets and boiled for 10 minutes.

7 Results

7.1 Stimulation and inhibition of PKC

7.1.1 PMA-dependent stimulation of PKC

PKC was shown to be activated by Phorbol-12-myristat-13acetat (PMA). The concentration of PMA for the best activation was evaluated by titrating PMA in T24 cells from 10 nM to 1 μM, as shown in the Western blot in Figure 6, panel A. Since PMA is solved in DMSO, the T24 cells in the control were incubated with 10 μl DMSO. The cells were incubated for 1 hour. The change of the protein level of p-PKC indicates the activation of PKC, and was detected with p-PKC antibody. Figure 6, panel B shows the quantification of the experiments presented in panel A. The increase of p-PKC shows that PKC was activated upon treatment with 10 nM, 50 nM, 100 nM, 200 nM PMA. Whereas the decreased protein levels of p-PKC with 400 nM, 600 nM, 800 nM and 1 μM indicated, that there is no activation of PKC.

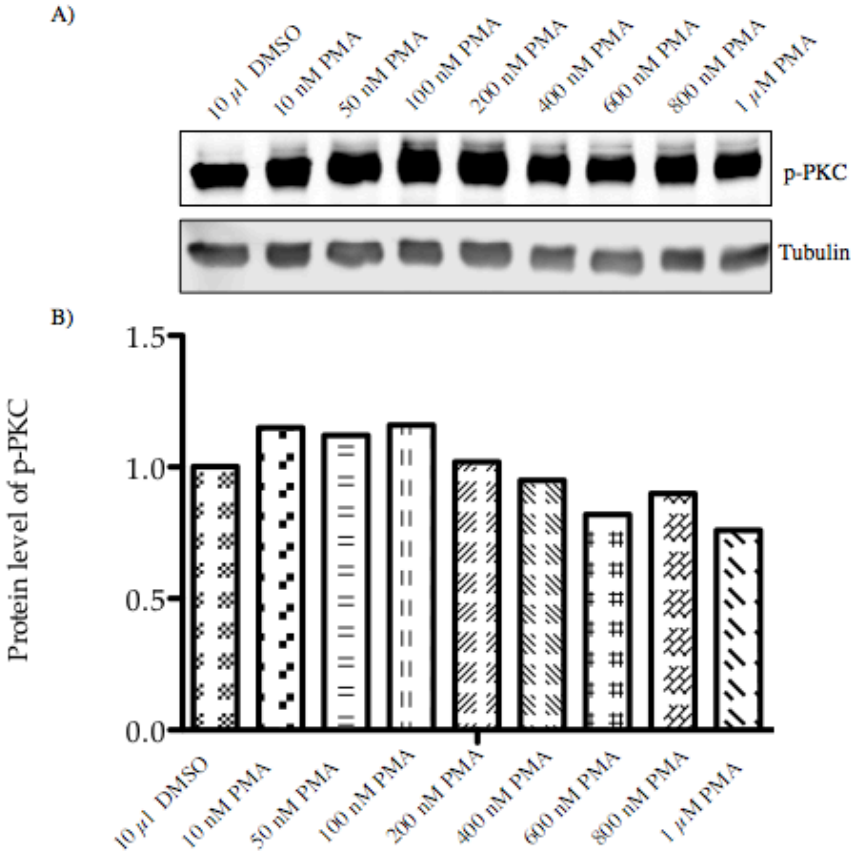

Figure 6: Titration of PMA in T24 cells. *(A) PMA was titrated from 10 nM to 1 µM as indicated, the cells were treated for 1 hour. The stimulation of PKC was detected with a p-PKC antibody. (B) Quantification of the result shown in A. The levels of p-PKC were normalized to tubulin.*

7.1.2 PMA activation efficiency decreases with time in T24 cells

T24 cells were treated with 100 nM PMA for 1 to 5 hours and as control treated with DMSO. The activation of PKC is shown in Figure 7, panel A. Panel B shows the quantification of the protein levels of p-PKC. The result of this quantification indicated that the best activation of PKC is upon treatment for 1 hour. From 2 to 5 hours no change of the level of p-PKC could be observed compared to the control group or 2 hours of treatment. Upon 3 to 5 hours of treatment the activation of PKC even decreased.

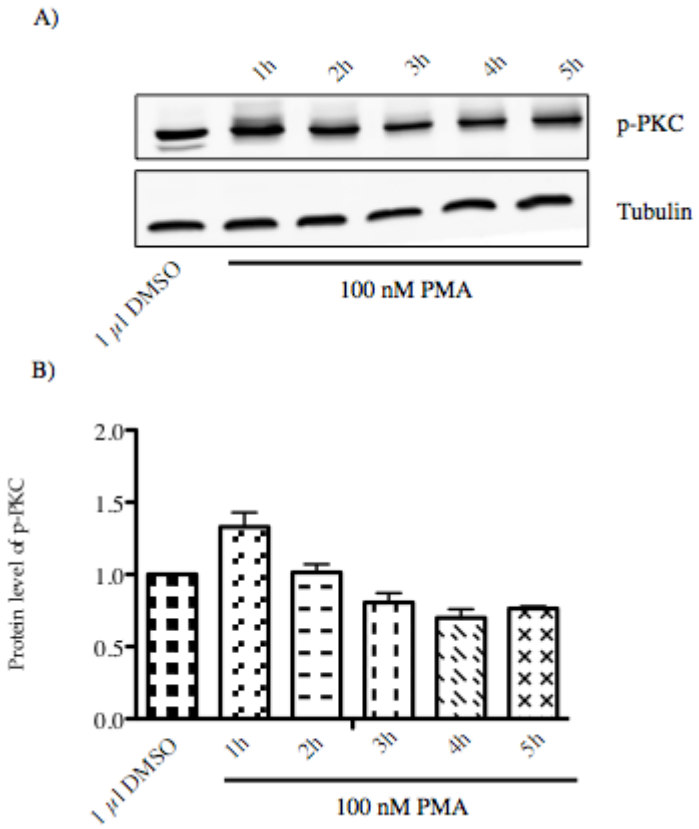

Figure 7: Kinetic of PMA in T24 cells*. (A) The T24 cells were treated with 100 nM PMA for 1 to 5 hours. The stimulation was detected with p-PKC antibody. (B) Shown is the quantification of the results from (A). The levels of p-PKC were normalized to tubulin.*

7.1.3 PMA activation efficiency decreases with time in HEK293T cells

The same experiment was performed by using HEK293T cells. The cells were treated with 200 nM PMA for 1, 2, 4 and 6 hours. The activation of PKC is shown in Figure 8, panel A. Panel B shows the quantification of the protein levels of p-PKC. A significant increase of p-PKC was observed after 1 hour of treatment compared to the control. After 2 hours only a slight activation could be detected, whereas after 4 and 6 hours of treatment no activation or even a decrease of p-PKC was observed.

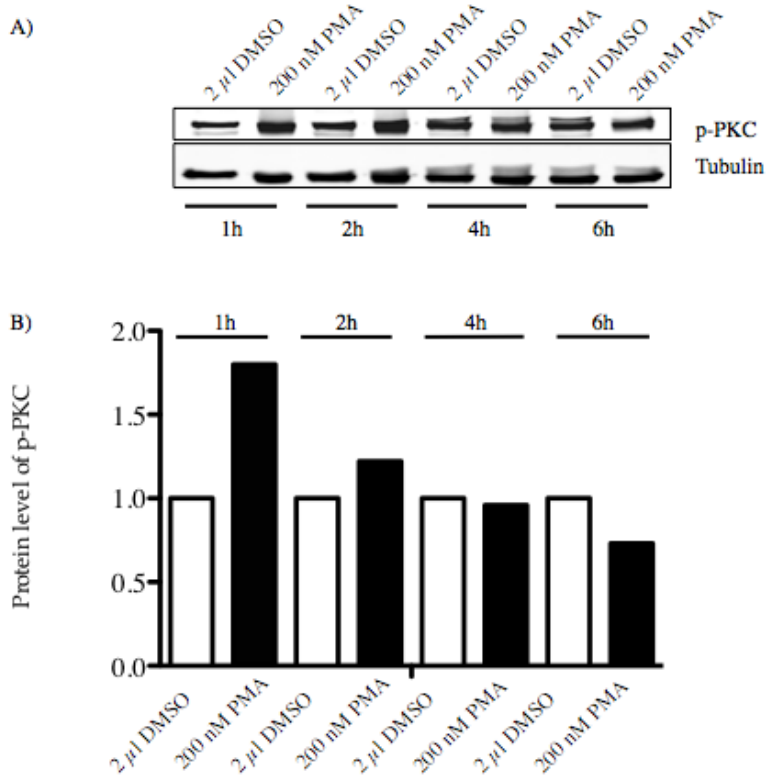

Figure 8: Kinetic of PMA in HEK293T cells. *(A) PKC was stimulated with 200 nM PMA for 1 to 6 hours as indicated. The stimulation was detected with a p-PKC antibody. (B) Shown is the quantification of the results presented in (A). The protein levels of p-PKC were normalized to tubulin.*

7.2 DNA polymerase β activation upon PMA treatment

7.2.1 Treatment with PMA leads to increased protein levels of DNA polymerase β in T24 cells

Next the change of the protein level of Pol β was evaluated in T24 cells. The cells were treated with different PMA concentrations for 1 hour. Figure 9, panel A shows the Pol β and p-CREB levels upon treatment. p-CREB as a downstream target of PKC was used as indicator for the activation of PKC. The quantification of panel B shows, that the activation of PKC with PMA worked with 100 nM, 200 nM or 400 nM PMA. Panel C shows the quantification of the change in Pol β protein levels upon treatment with PMA compared to the control group. The protein levels of Pol β increased upon treatment in general, but the highest increase was observed after 1 hour treatment with 200 nM PMA.

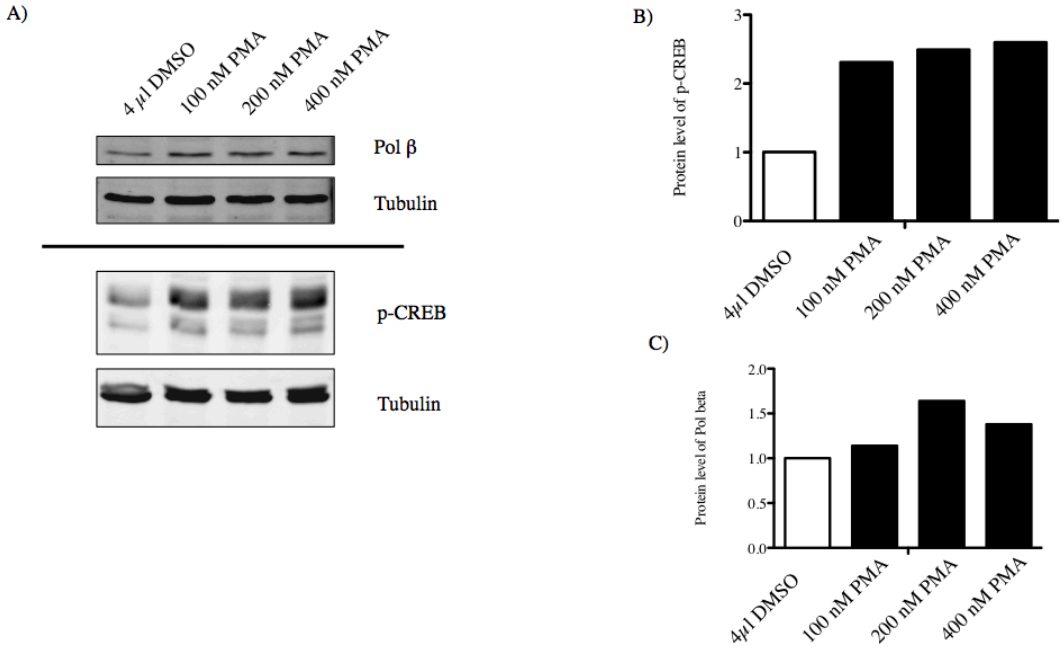

Figure 9: DNA polymerase β protein levels increase upon PKC stimulation in T24 cells. *(A) The cells were treated with either 100 nM PMA, 200 nM PMA or 400 nM PMA and the protein levels evaluated by Western blotting. The stimulation of PKC was detected with p-CREB antibody (B) The stimulation of PKC in (A) was evaluated by normalization of the protein levels of p-PKC to tubulin. (C) Quantification of the protein levels of Pol β shown in (A), the protein levels were normalized to tubulin.*

7.2.2 Treatment with PMA leads to increased protein levels of DNA polymerase β in HEK293T cells

Changes of the protein levels of Pol β were examined, upon treatment of HEK293T with PMA or as a control with DMSO (Figure 10, panel A). The HEK293T cells were either treated for 2 or 4 hours. In the quantification shown in panel C, the differences in the Pol β protein levels between the treated and non-treated HEK293T cells is clearly visible. The cells treated with PMA had increased Pol β protein levels. After 2 hours treatment the protein levels were slightly higher compared to 4 hours of treatment.

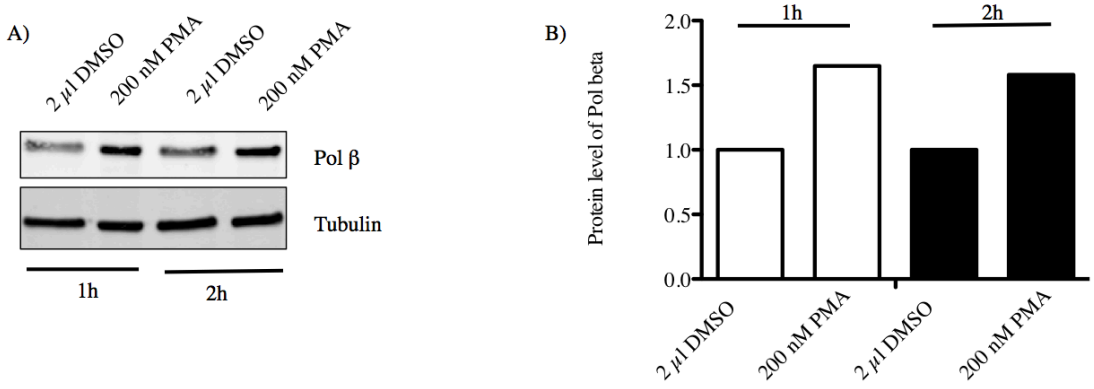

Figure 10: DNA polymerase β protein levels increase upon PKC stimulation in HEK293T cells*. (A) HEK293T cells were treated with 200 nM PMA and the protein levels of Pol β evaluated by Western blotting. (C) Quantification of the result shown in (A). The Pol β protein levels were normalized to tubulin.*

7.3 Characterization of the interaction between DNA polymerase β and XRCC1

7.3.1 Overexpression of XRCC1 in HEK293T cells

For the characterization of the interaction of Pol β with XRCC1, HEK293T cells were transfected with either 1 µg or 3 µg of XRCC1. Figure 11 shows the Western blot of whole cell extracts, confirming the overexpression of XRCC1. Under both conditions the transfection worked. The lysates of the transfected HEK293T cells were used for further experiments as described in the following.

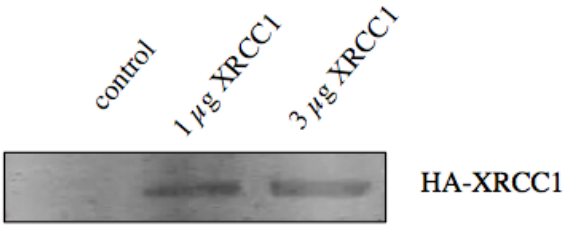

Figure 11: Transfection of HEK293T cells with HA-XRCC1. *HEK293T cells were either transfected with 1 µg or 3 µg of DNA. 40 µg of the whole cell extract was loaded on a SDS-PAGE and XRCC1 was detected by Western blotting with HA-antibody.*

7.3.2 DNA polymerase β WT interacts with XRCC1 in a HIS-pulldown

For the investigation of the interaction of Pol β with XRCC1 a HIS-pulldown was performed, using HIS-tagged Pol β WT bound to Ni-beads (Figure 12). Different NP-40% concentrations (0.1, 0.2 and 0.4 %) were used. To prove, that there is no unspecific binding of XRCC1 to the Ni-beads, a control without Pol β was included. All of the used concentrations of NP-40 eliminated unspecific binding of XRCC1 to the Ni-beads, while Pol β bound beads co-immunoprecipitated with XRCC1. This result confirmed the interaction between the two proteins. The control in the last two lanes was performed without XRCC1 to confirm an equal binding of Pol β to the Ni-beads under different conditions.

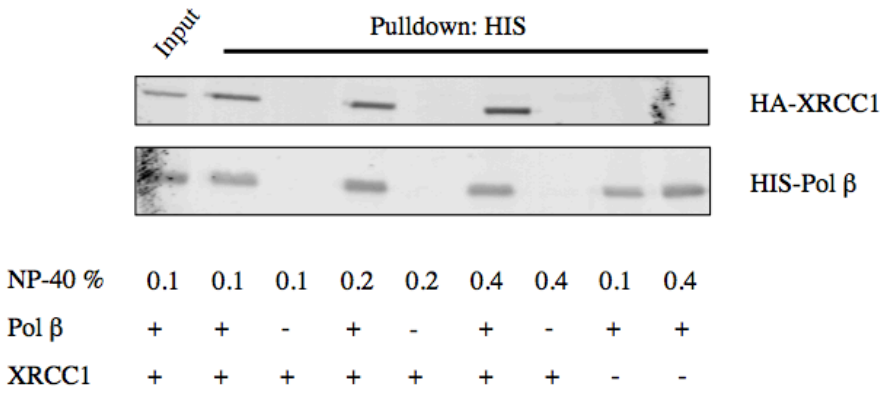

Figure 12 DNA polymerase β WT interacts with XRCC1. *HIS-pulldown of HA-XRCC1 with HIS-Pol β WT. The recombinant and purified proteins were incubated with Ni-beads. The input corresponds to 10 % of the used protein.*

7.3.3 Differences in interaction of DNA polymerase β WT and the phosphorylation deficient point mutants with XRCC1 in a HIS pulldown

After the binding of Pol β WT to XRCC1 was confirmed the differences in binding of Pol β WT and the phosphoablated mutants to XRCC1 was investigated. Again a HIS-pulldown was performed using HIS-tagged Pol β WT and HIS-tagged Pol β mutants bound to Ni-beads. Figure 13, panel A shows the result of the pulldown. The negative control without Pol β does not show any XRCC1 unspecific bound to the Ni-beads. The Input contains Pol β and XRCC1. The experiment was performed with 0.2 % NP-40. Panel B shows the quantification of the different binding capacities of the Pol β mutants compared to the Pol β WT. These preliminary data implied, that the Pol β S55A mutant binds XRCC1 better than the Pol β WT. The other Pol β mutants T10A, S30A, S44A, T67A and S180A bind less XRCC1 compared to Pol β WT.

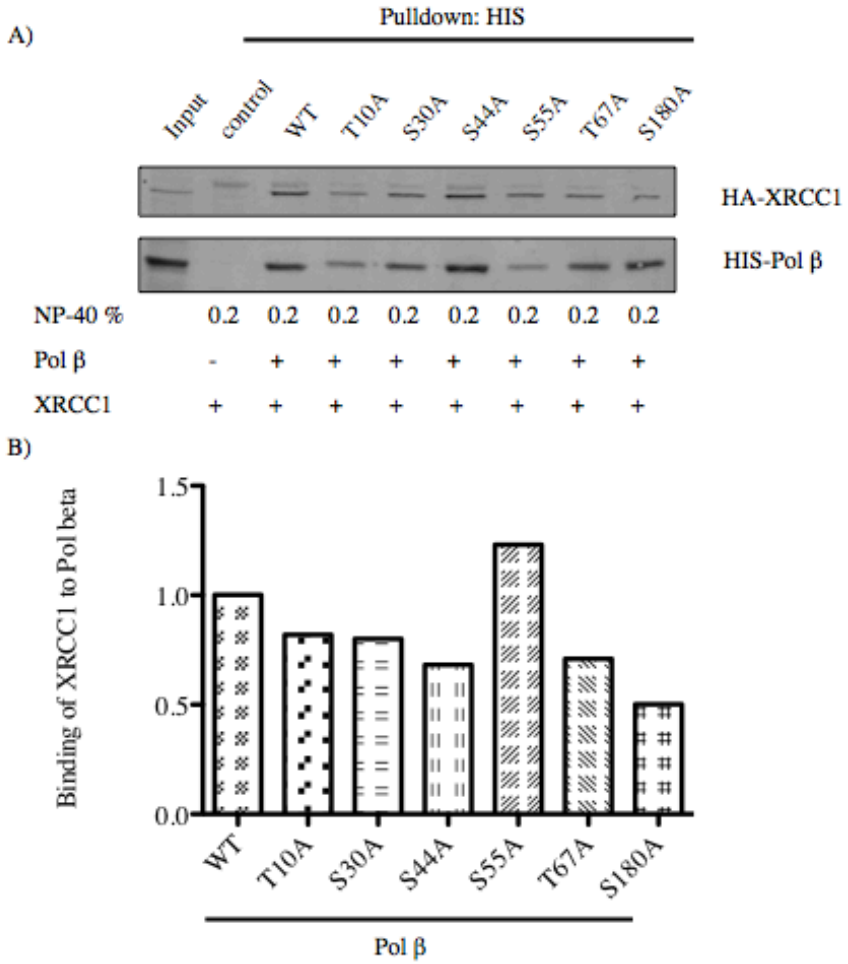

Figure 13: Pulldown of DNA polymerase β mutants with XRCC1*. (A). HIS-pulldown of HA-XRCC1 with HIS-Pol β WT and HIS-Pol β mutants as indicated. The recombinant and purified proteins were incubated with Ni-beads. The input corresponds to 10 % of the used protein. (B) Quantification of the result from (A). The protein levels of XRCC1 were normalized to Pol β bound to the beads.*

7.3.4 Interaction of DNA polymerase β with XRCC1 increases upon PKC stimulation with PMA

After the interaction of Pol β WT with XRCC1 was investigated *in vitro* the result was also confirmed in a co-immunoprecipitation (co-IP) experiment. HEK293T cells were transfected with Pol β and XRCC1 and afterwards the transfected cells were treated with 200 nM PMA or 200 nM PMA + 100 nM ro 32-0432 (a PKC inhibitor). As a control cells were treated with DMSO. Figure 14, panel A shows the Western blot of the co-IP. The IP was performed with a Flag antibody. In the left part of panel A the input is shown confirming the stimulation of PKC (quantification in panel B). Upon treatment of the cells with PMA + ro 32-0432 the activation of PKC could be reduced. The right part of Panel A shows the interaction of Pol β with XRCC1. Panel C shows the change of the binding of Pol β to XRCC1. When the cells were treated with PMA and PMA + ro 32-0432, the binding of Pol β to XRCC1 was increased compared to the untreated cells. The fact that the inhibition of PKC was incomplete might explain, why the interaction of Pol β with XRCC1 remains unchanged.

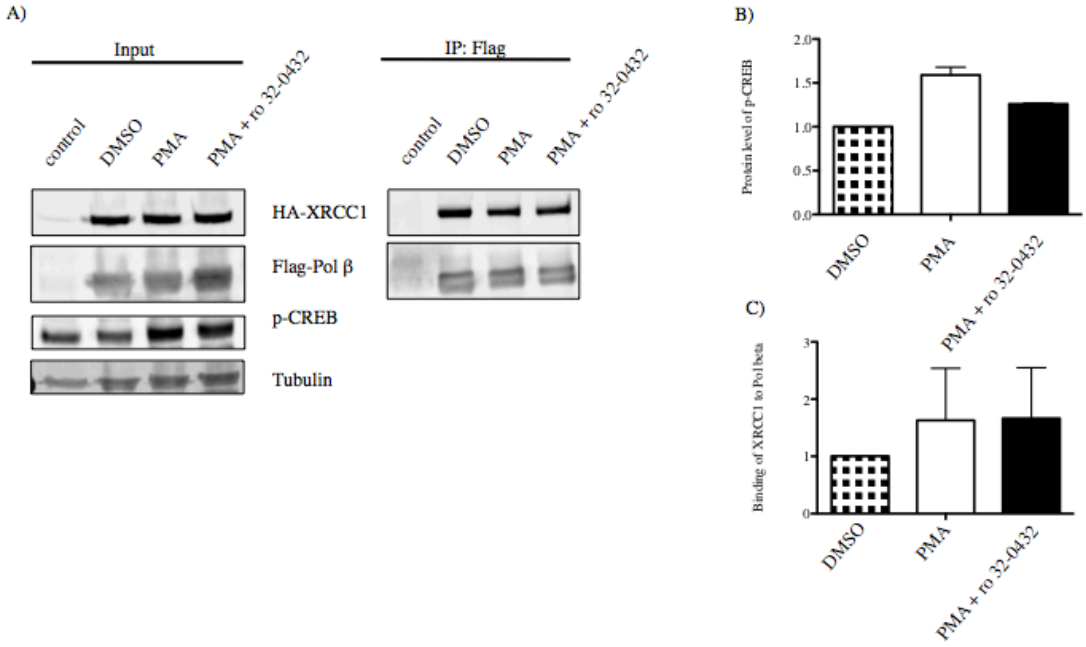

Figure 14: PKC stimulation increases the interaction between DNA polymerase β and XRCC1. *(A) Co-immunoprecipitation of HA-XRCC1 with Flag-Pol β WT. PKC was stimulated with 200 nM PMA for 1 hour or inhibited with 200 nM PMA + 100 nM ro 32-0432 for 1 hour. Whole cell extracts containing the overexpressed proteins were incubated with Flag AB coupled to beads. The input corresponds to 5 % of the used proteins. (B) Quantification of the stimulation of PKC, shown in (A). The protein levels of p-CREB were normalized to Pol β. (C) Quantification of the binding of XRCC1 to Pol β, shown in (A). The protein levels of XRCC1 were normalized to Pol β.*

7.4 Characterization of the interaction between DNA polymerase β and PCNA

7.4.1 Interaction of DNA polymerase β with PCNA decreases upon PKC stimulation with PMA

First the interaction of PCNA with Pol β upon PKC stimulation or inhibition was confirmed in a co-IP. HEK293T cells were transfected with Pol β and XRCC1 and the cells were treated with either 200 nM PMA or 200 nM PMA + 100 nM ro 32-0432 for 1 hour. The binding of Pol β to PCNA in the treated cells was compared to the binding of Pol β to PCNA in untreated HEK293T cells (Figure 16). The stimulation of PKC was evaluated with p-CREB (quantification in panel B). The protein levels of p-CREB increased upon stimulation with PMA and decreased, upon treatment with ro 32-0432. In the right part of panel A the result of the IP is shown. Panel C indicates that the binding of Pol β to PCNA significantly decreases upon stimulation of PKC, when compared to the untreated HEK293T cells.

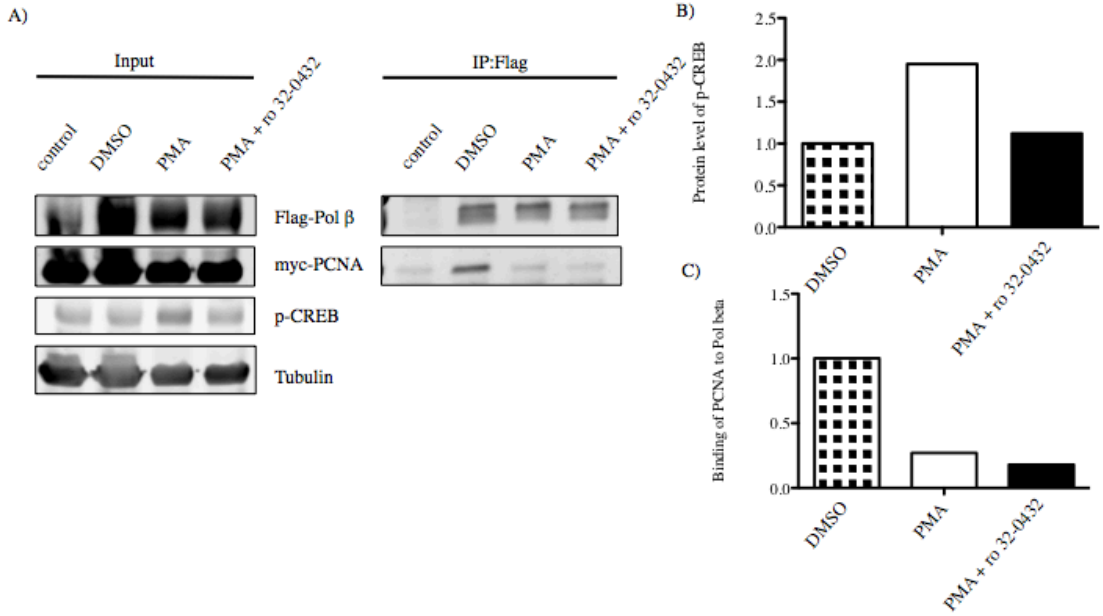

Figure 15: PKC stimulation increases the interaction between* DNA polymerase *β and PCNA. *(A) Co-immunoprecipitation of myc-PCNA with Flag-Pol β WT. PKC was stimulated with 200 nM PMA for 1 hour or inhibited with 200 nM PMA + 100 nM ro 32-0432 for 1 hour. Whole cell extracts containing the overexpressed proteins were incubated with Flag AB coupled to beads. The input corresponds to 5 % of the used proteins. (B) Quantification of the stimulation of PKC, shown in (A). The protein levels of p-CREB were normalized to Pol β. (C) Quantification of the binding of PCNA to Pol β, shown in (A). The protein levels of PCNA were normalized to Pol β.*

8 Discussion

Posttranslational modifications play key roles in the BER complex formation, thereby modulating the specificity and efficiency of the repair of damaged DNA (31). The aim of this thesis was to investigate the effect that phosphorylation by PKC has on Pol β. In earlier work it was shown *in vitro*, that phosphorylation of Pol β by PKC had no influence on its activity on a single nucleotide gap template (1). Therefore we wanted to know, whether the phosphorylation of Pol β by PKC has any influence on the stability or the binding to important interaction partners in BER, like XRCC1 and PCNA. Phosphorylation of Pol β by PKC can be regulated by PMA that stimulates PKC, or with ro 32-0432 that inhibits PKC. In preliminary experiments optimal conditions for the stimulation of PKC were examined. To investigate what effect the phosphorylation of Pol β by PKC has, the protein levels of Pol β in cells treated with PMA were determined. The results indicated a correlation between the stimulation of PKC by PMA and enhanced protein levels of Pol β. The data obtained indicated that phosphorylation of Pol β by PKC leads to increased Pol β levels and are therefore a hint, that this essential BER protein is stabilized by phosphorylation. These experiments were performed in different cell lines (T24 cells and HEK293T cells) with the same outcome, implicating a general and cell line independent effect (Figures 9 and 10).

In further studies the binding of Pol β in dependency on phosphorylation was investigated to different interaction partners. In a preliminary experiment, a HIS-pulldown was performed, showing that HIS-tagged Pol β WT interacts with XRCC1 in whole cell extracts (Figure 12). This result is in accordance with previous studies showing that XRCC1 stabilizes Pol β and is a recruiting factor of Pol β to damaged DNA (78, 80). Further it was investigated whether the PKC mediated phosphorylation of Pol β has any influence on its binding to XRCC1. Therefore the HIS-pulldown was repeated using HIS-tagged Pol β WT and the HIS-tagged phosphoablated point mutants of Pol β (T10A, S30A, S44A, S55A, T67A, S180A). Binding decreased of Pols β T10A, S30A, S44A, T67A and S180A to XRCC1 compared to Pol β WT to XRCC1 (Figure 13). Bhattacharyya et al. investigated the binding of a Pol β deletion construct (delta 208-236) to XRCC1. They found, that the Pol β mutant binds XRCC1 better compared to the full-length construct, but was less able to participate in DNA synthesis. The results for the S55A mutant investigated in this thesis work are comparable to those previous observations in regard to increase in its binding ability (Figure 13, panel B) and decrease in its processivity in a single nucleotide gap primer/template assay (1). It was suggested, that this deletion might lead to genetic instability and cancerogenesis, which calls for future investigation of these particular mutations, especially with regards to single strand breaks, genomic instability and cancerogenesis (129).

This need was further highlighted by the fact, that the Pol β mutant S44F was shown to play a role in colon cancer (130). Surprisingly, in experiments of this thesis work Pol β mutant S44A binds less to XRCC1 compared with the Pol β WT, quite in contrast to the above described S55A, therefore suggesting a different mechanism for the increase in genomic instability. Since Pol β was shown to be more stable if present in a complex, it might be speculated that the decreased binding of this mutant to XRCC1 (Figure 13, panel B) contributes to a decreased stability of Pol β and faster degradation of the protein and this therefore might suggest an onset of mutations

(130). Also the Pol β mutants T10A, S30A, T67A and S180 show a decreased binding to XRCC1 indicating that in general the phosphorylation sites of Pol β are important for its binding to XRCC1. In summary these result point in the direction, that phosphorylation of Pol β plays an important role in the interaction with XRCC1, although none of the Pol β mutants is directly located at the predicted binding site of Pol β to XRCC1 (Figure 16).

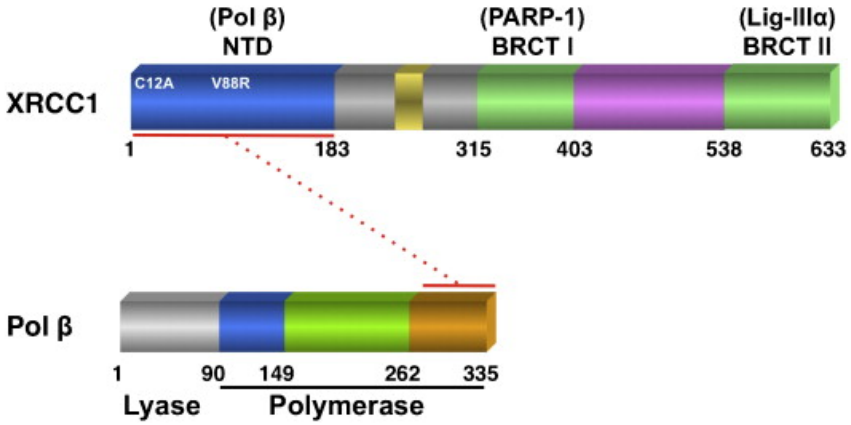

Figure 16: Domain organization of mouse XRCC1 and DNA polymerase β proteins. *The N terminal domain of XRCC1 interacts with the C terminal end of the polymerase domain of Pol β (dotted red line) (adapted from reference (131)).*

Since it was shown, that the phosphorylation sites of Pol β can modulate its binding to XRCC1 *in vitro*, it was investigated, whether stimulation of PKC has an influence on the binding of Pol β to XRCC1 *in vivo*. Therefore HEK293T cells were transfected with Pol β WT and XRCC1. The transfected cells were treated with either the PKC activator PMA or with the PKC inhibitor ro 32-0432 (Figure 14, panel B). The binding of Pol β WT to XRCC1 was increased upon stimulation of PKC (Figure 14, panel C). This effect could not be completely rescued by inhibiting PKC with ro 32-0432, likely because inhibition itself was incomplete. These data must be seen in relation to DNA damage repair, that implies that higher levels of phosphorylated Pol β lead to an increase in binding to XRCC1. Therefore the BER complex becomes stabilized and recruitment of Pol β to damaged DNA is enhanced, thus promoting DNA repair (78, 80). Furthermore it is known, that PKC and CREB are survival markers of the cell. Irradiated primary cells activate the DNA damage check-point response and cell cycle arrest, which either leads to apoptosis or senescence. Bluwstein et al. irradiated primary cells and showed, that down-regulation of PKC in primary human fibroblasts leads to irradiation-dependent down-regulation of CREB phosphorylation and therefore causes proliferation stop and apoptosis. With these findings it was suggested, that an irradiation-dependent PKC up-regulation prevents irradiation-dependent apoptosis (132). These findings have to be further investigated in context of cancer treatment.

PCNA is a key player in LP-BER. It encircles the damaged DNA and recruits BER proteins, mainly the replicative Pols δ and ε. Former studies already showed a direct dependency of the binding capacities of Pol β to PCNA on PTMs. El-Andaloussi et al. showed, that methylation of Pol β by arginine methylransferase 1 prevents binding of Pol β to PCNA. In this context it was interesting to investigate the PTMs of Pol β in

more detail to get a conclusive picture of its regulation. Therefore the binding of Pol β WT to PCNA changes upon stimulation or inhibition of PKC was investigated. HEK293T cells were transfected with Pol β WT and PCNA and the transfected cells treated with either PMA or ro 32-0432. In Figure 15 the effect on the binding of PCNA to Pol β is shown, indicating that it decreases significantly upon activation of PKC (Figure 15, panel C). Figure 18 shows, that Pol β has three PCNA interacting motifs (PIM). The first PIM is located between aa 136 - 143, the second between aa 217 - 223 and the third between aa 246 - 272 (Figure 17). The PKC dependent phosphorylation sites that were identified are not located within one of these PIMs. Therefore it is concluded, that phosphorylation does not directly compete with the binding of Pol β to PCNA, but might change its secondary structure.

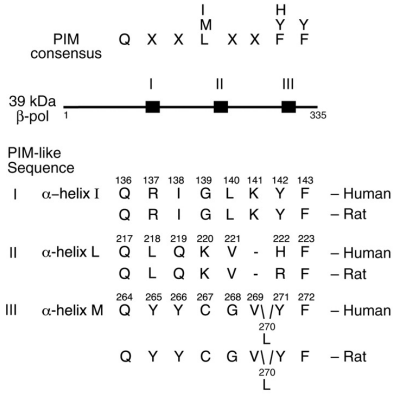

Figure 17: Sequence alignments illustrating the presence of three sequences in DNA polymerase β resembling the consensus PCNA interacting motif or PIM. *Three PIM-like sequences were identified in Pol β that resemble the consensus PIM, consisting of the sequence QXX(h)XX(a)(a), where h is a moderately hydrophobic side chain, a is a hydrophobic side chain and X is any residue. The figure compares the three PIM-like sequences in human and rat Pol β. The PIM-like sequences I, II, and III are aligned within the 39-kDa 335-residue Pol β sequence (adapted from reference (93)).*

It was shown in this study that the crucial impact of PTMs on the Pol β enzyme stability and its binding to important interaction partners. These findings give a deeper insights into the regulation of DNA repair and have therefore to be further investigated in the association with cancers. Finally it might point to future directions for cancer therapies.

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